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PRINCIPAL INVESTIGATOR: Tracy Vargo-Gogola, Ph.D.
Jeffrey M. Rosen, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Tracy Vargo-Gogola, Ph.D.
Jeffrey M. Rosen, Ph.D.

E-Mail: tracyv@bcm.tmc.edu

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14. ABSTRACT

We have developed a tetracycline-regulatable p190-B RhoGAP overexpressing mouse model to investigate the role of p190-B in mammary gland development and breast cancer progression. We have now shown that overexpression of p190-B in the developing mammary gland disrupts terminal end bud (TEB) architecture, increases branching, delays ductal elongation, and leads to disorganization of the ductal tree. Several cellular and molecular changes were detected in the p190-B overexpressing TEBs, all of which may contribute to the disruption in ductal morphogenesis. Alterations in the insulin like growth factor (IGF) signaling pathway, pronounced changes in the microenvironment, and discontinuity of the myoepithelial cell layer was detected in the p190-B overexpressing TEBs. Furthermore, overexpression of p190-B during pregnancy results in hyperplastic lesions. This report is the first to demonstrate that overexpression of a RhoGAP in vivo results in neoplastic growth. These results are presented in the manuscript entitled "P190-B overexpression disrupts ductal morphogenesis in the developing mammary gland," which is attached as Appendix 1.

15. SUBJECT TERMS
P190-B RhoGAP, terminal end buds, ductal morphogenesis, mammary gland development, tumor biology, cell signaling

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Introduction

During the 12 months of this reporting period, our efforts have been focused on completing examination of the effects of p190-B overexpression in the developing mammary gland. Attached as Appendix 1 is a manuscript describing the effects of p190-B overexpression in the developing virgin, pregnant, and lactating mammary gland. After obtaining revisions from all authors the manuscript will be submitted for publication in October 2005. Investigation of the effects of p190-B overexpression in the involuting mammary gland is still in progress, and the results to date are detailed below.

Body

Task 1. To elucidate the role of p190-B in mammary gland development in p190-B deficient and tetracycline (tet)-regulatable p190-B overexpressing mice (Months 1-24).

To examine the consequences of p190-B overexpression during mammary gland involution, p190-B/MMTV-rtTA bigenic female mice at eight weeks of age were bred to wildtype male mice. Age-matched MMTV-rtTA and wildtype female mice served as controls. Doxycycline (Dox) treatment to induce transgene expression was started on the first day of pregnancy and continued throughout lactation and involution. Control mice were also treated with Dox to rule out any possible non-specific effects of the doxycycline treatment. The mice were allowed to lactate for 21 days at which time the pups were weaned. Fourteen days after natural weaning mammary glands were dissected and examined by whole-mount mammary gland and histological analysis of hematoxylin and eosin stained tissue sections.

Interestingly, the p190-B overexpressing mammary glands showed abnormal ductal histology following involution. In particular, the walls of the ducts appeared thicker and contained lipid-like droplets underlying the luminal epithelial cell layer (Figure 1). To determine whether both luminal and myoepithelial cell types were present in the aberrant ducts, immunostaining for a luminal cell marker, E-cadherin, and a myoepithelial marker, smooth muscle actin, was performed. While both cell types were still present in the abnormal ducts, expression of these two proteins was altered (Figure 2). Expression of E-cadherin surrounding the lipid-like droplets was no longer localized to the basal-lateral region of the cell, but was now located at the apical surface (Figure 2B). Smooth muscle actin expression, which normally forms a nearly continuous layer within the myoepithelial cells underlying the luminal cells, was disrupted in the abnormal ducts and showed a punctate staining pattern (Figure 2D). These data suggest that p190-B overexpression during involution alters the ductal architecture potentially by changing cell polarity in the luminal cells and myoepithelial cell function.

Although these droplets are lipid-like in appearance it is not clear whether they are infiltrating adipocytes, or accumulated lipid droplets or mucin. Immunostaining for several markers including mucin 1 and the lipid droplet marker adipophilin has proved difficult as these droplets show high background florescence with these as well as a number of other antibodies. Thus, this alteration in the ductal structure of the p190-B overexpressing mammary glands requires further examination.

As described in the attached manuscript, hyperplastic lesions were detected in the mammary glands of p190-B overexpressing mice during pregnancy. This was an exciting result as to our knowledge alteration in the expression of a RhoGAP has not

been shown to have this effect *in vivo*. To determine whether these lesions will progress over time a long-term study in which the effects of persistent p190-B transgene expression in multiparous mice is currently underway, and the mice have now been through two pregnancies. After the third round of pregnancy and involution, mammary glands will be collected and analyzed for neoplastic growths. Thus far, lactation in the p190-B overexpressing mice appears normal since they have been able to support litters of 6 or more pups.

Task 2. To investigate the role of p190-B in breast cancer progression using both p190-B loss and gain of function mouse models (Months 12-36).

The p190-B null mice were generated on a C57Bl/6 genetic background, which has been reported to be resistant to mammary gland tumorigenesis. Therefore, these mice are being back-crossed into an FVB genetic background before they will be bred to the MMTV-ErbB2 mice. We have recently begun to obtain trigenic p190-B/MMTV-rtTA/MMTV-ErbB2 female mice. When these mice reach 8 weeks of age Dox treatment to induce p190-B transgene expression will be started. As more trigenic mice are obtained they will be added to the study, which will continue for the next 12-18 months.

Task 3. To delineate the molecular mechanism by which p190-B facilitates tumor cell migration and invasion using siRNA to knockdown p190-B expression in MCF7 breast cancer cells (Months 12-36).

Thus far, these studies have not yielded insight into the unique roles of p190-B in migration and invasion in the MCF7 cell culture model, and interestingly, they have revealed a surprising overlap in the activities of the p190-A and p190-B proteins. We have now shown that p190-A is also expressed in these cells, and it shows an identical pattern of phosphorylation in response to IGF-1 and estrogen treatment (Figures 3A-E). Furthermore, these proteins co-immunoprecipitate in extracts prepared from both breast cancer cell lines as well as normal mammary gland (Figure 3G-F). In addition, when siRNA is used to knock down p190-B expression, p190-A phosphorylation in response to IGF-1 and estrogen treatment appears even stronger (Figure 3C). When both p190-B and p190-A are knocked down at the same time the cells undergo apoptosis (data not shown). While this demonstrates a critical role for the function of these RhoGAP proteins in MCF7 cell survival delineating unique activities of these proteins in this cell culture model is proving to be challenging. We are currently trying to identify the other proteins within the complex that contains p190-B and p190-A in order to further elucidate the roles of these two proteins in mammary gland development and breast cancer progression.

Key Research Accomplishments

- A manuscript describing the effects of p190-B overexpression in the developing virgin, pregnant, and lactating mammary gland has been generated.
- P190-B RhoGAP overexpression during pregnancy leads to hyperplastic lesions.

- A few trigenic tet-regulatable p190-B/MMTV-rtTA/MMTV-ErbB2 mice have been obtained so we can begin to examine the effects of p190-B overexpression on tumor latency and growth.

Reportable Outcomes

A manuscript entitled "P190-B RhoGAP overexpression disrupts ductal morphogenesis in the developing mammary gland" has been prepared.

Conclusions

We have now completed an investigation of the effects of p190-B RhoGAP overexpression in the developing virgin, pregnant, and lactating mammary gland. These studies have revealed interesting results demonstrating that p190-B is critical for terminal end bud morphology. Overexpression of p190-B in the developing gland leads to delayed ductal elongation, disorganization of the ductal tree, and increased branching. These phenotypes may be the result of altered IGF signaling, disruption of the myoepithelial cell layer surrounding the terminal end buds, and dramatic changes in the adjacent microenvironment. Importantly, overexpression of p190-B resulted in hyperplastic lesions during pregnancy. This result is one of the first examples in which overexpression of a RhoGAP is shown to lead to neoplastic changes *in vivo*.

Appendices

See attached manuscript entitled "P190-B RhoGAP overexpression disrupts ductal morphogenesis in the developing mammary gland."

Contact information:
Tracy Vargo-Gogola, Ph.D.
Department of Molecular and Cellular Biology
Baylor College of Medicine, M637
Houston, TX 77030
Phone 713-798-6211
Fax 713-798-8012

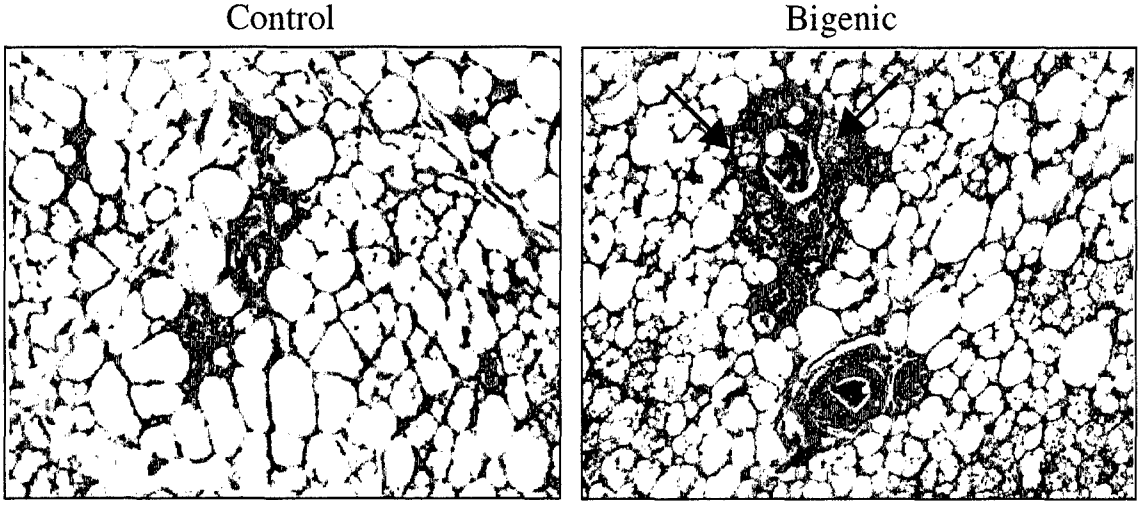

Figure 1: Hematoxylin and eosin stained tissue sections from involuting p190-B overexpressing (Bigenic) and control mammary glands. Arrows point to abnormal lipid-like droplets in the walls of the aberrant ducts.

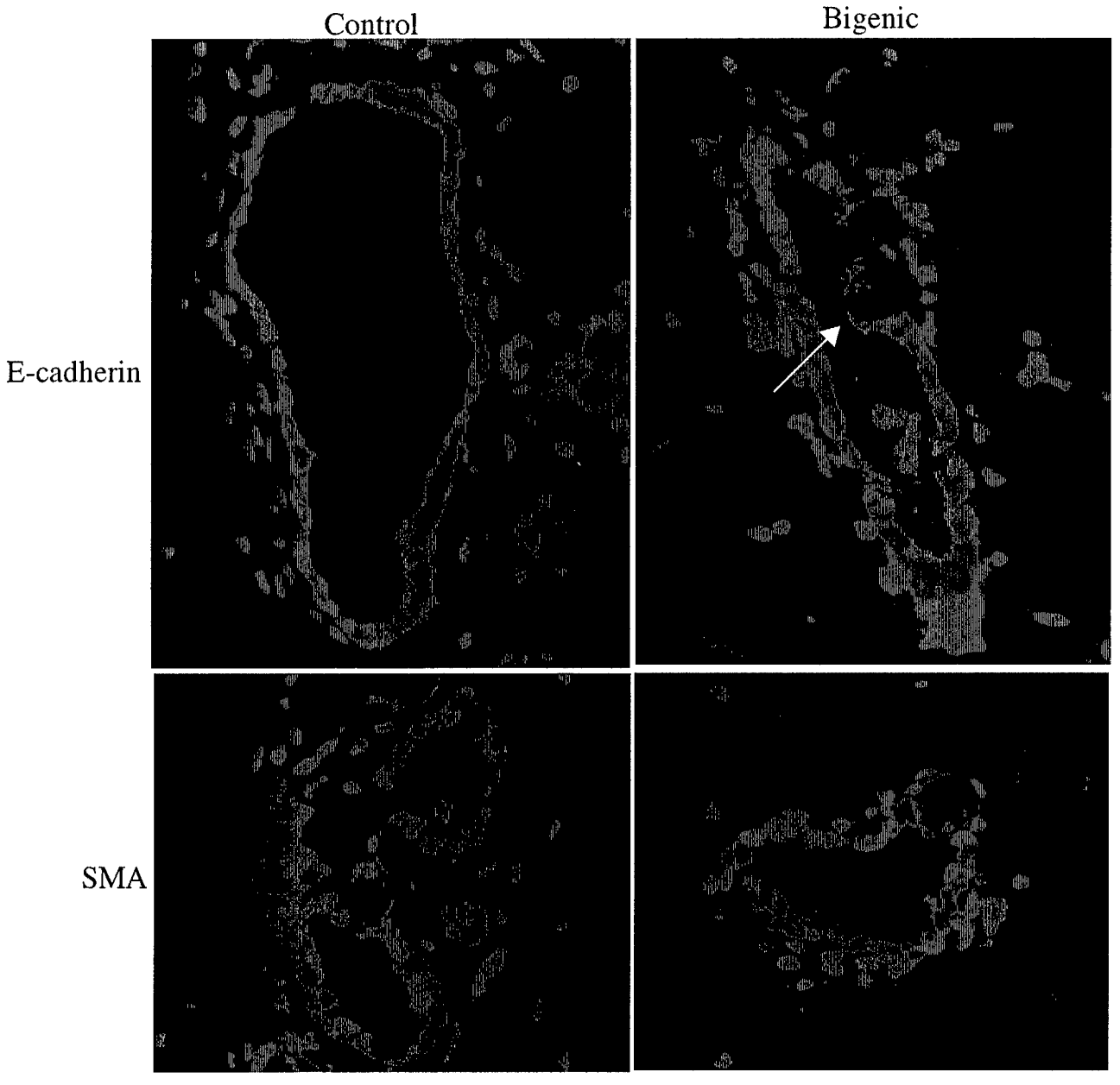

Figure 2: Immunostaining for E-cadherin and smooth muscle actin (SMA) in tissue sections from p190-B overexpressing (Bigenic) and control involuting mammary glands. The arrow in the top right panel indicates where E-cadherin staining is now localized to the apical surface of the duct. Note the punctate staining pattern of the SMA in the bigenic duct as compared to the control duct, which shows a nearly continuous layer of SMA underlying the luminal epithelial cells.

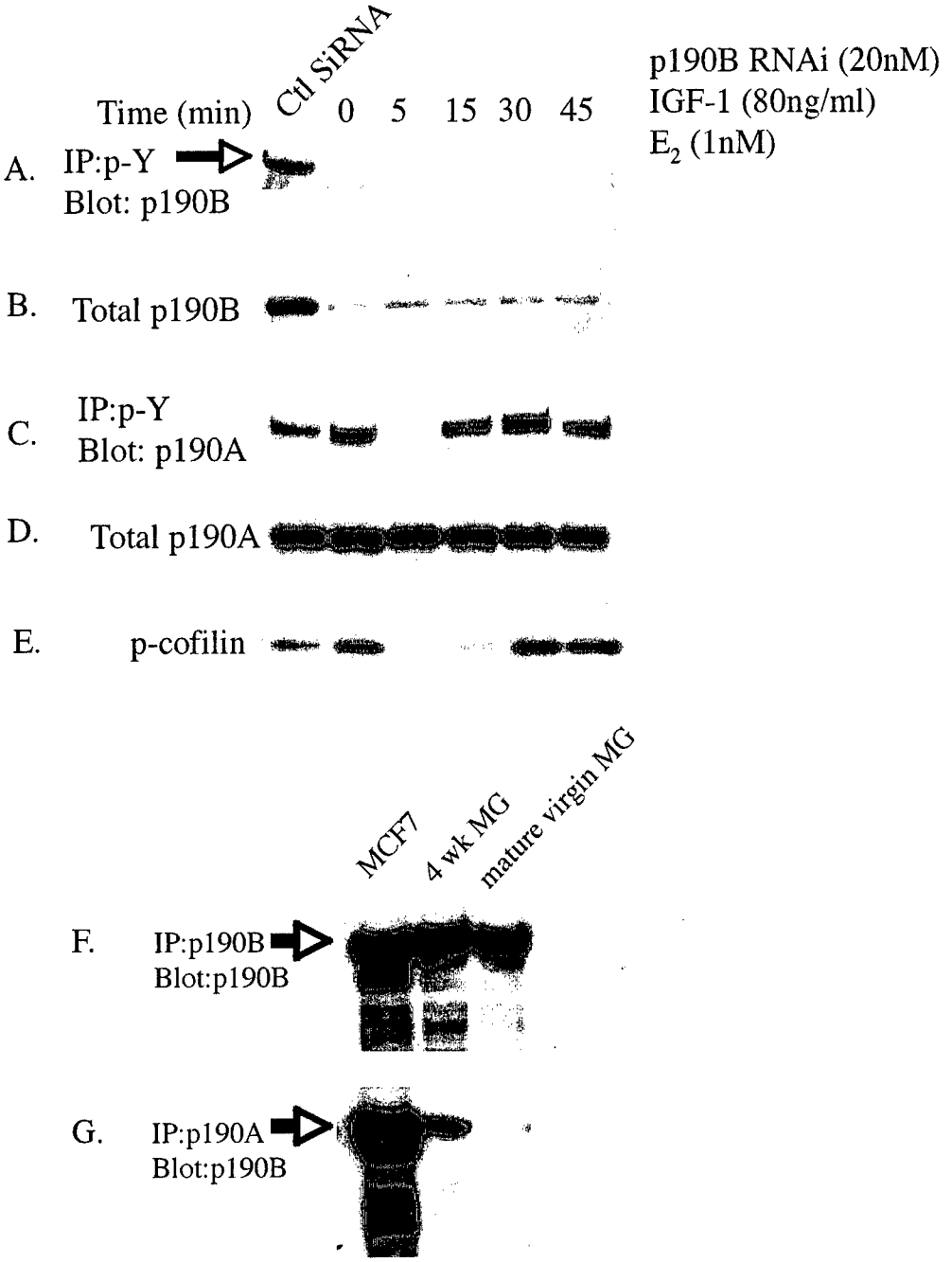

Figure 3: P190-A may compensate for loss of p190-B . SiRNA was used to specifically knockdown p190-B (panel B vs. panel D). Cells were then serum starved overnight and treated with IGF-1 and estrogen. Cell lysates were prepared at the indicated time points. A phospho-tyrosine antibody was used for immunoprecipitation followed by Western blotting for either p190-B or p190-A (panels A and C). P190-A shows an identical tyrosine phosphorylation pattern compared to p190-B in response to IGF-1 and estrogen treatment (see faint bands in panel A indicateed by the arrow), and this response is not altered when p190-B expression is knocked down by siRNA treatment. Phospho-cofilin is a downstream target of the Rho pathway, and its phosphorylation is unaltered by the loss of p190-B (panel E). P190-A and P190-B are in the same complex in MCF7 cells and in the developing mammary gland (MG) and mature virgin mammary gland as shown by co-immunoprecipitation and Western blotting (panels F and G).

APPENDIX

P190-B RhoGAP overexpression disrupts ductal morphogenesis and induces hyperplastic lesions in the developing mammary gland

Tracy Vargo-Gogola[1], Brandy M. Heckman[1], Lewis A. Chodosh[2], and Jeffrey M. Rosen[1*]

[1]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston TX, 77030
[2] Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia PA 19104

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*To whom correspondence and requests for reprints should be addressed:
Jeffrey M. Rosen, Ph.D.
C.C. Bell Professor of Molecular and Cellular Biology and Medicine
DeBakey Building, M638a
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030-3498
FAX 713-798-8012
Telephone 713-798-6210
Email: jrosen@bcm.tmc.edu

Abbreviations

AKT-PKB or protein kinase B
CSF-1-colony stimulating factor 1
Dox-doxycycline
ECM-extracellular matrix
iFGFR1-inducible fibroblast growth factor receptor 1
GAP-GTPase activating protein
H&E-hematoxylin and eosin
IGF-insulin like growth factor
IGF-IR-insulin like growth factor receptor I
IRS-insulin related substrate
MTB-MMTV-rtTA transgenic mice
ROK-Rho kinase
Tet-tetracycline
TEB-terminal end bud

Abstract

P190-B Rho GTPase activating protein (GAP) is essential for mammary gland development since p190-B deficiency prevents ductal morphogenesis. To investigate the role of p190-B during distinct stages of mammary gland development, tetracycline (tet)-regulatable p190-B overexpressing mice were generated. Short-term induction of p190-B in the developing mammary gland results in abnormal terminal end buds (TEBs) that exhibit aberrant budding off the neck, histological anomalies, and a markedly thickened stroma. Overexpression of p190-B throughout postnatal development results in increased branching, delayed ductal elongation, and disorganization of the ductal tree. Interestingly, overexpression of p190-B during pregnancy results in hyperplastic lesions. Several cellular and molecular alterations detected within the aberrant TEBs may contribute to these phenotypes. Signaling through the insulin-like growth factor (IGF) pathway is altered, and the myoepithelial cell layer is discontinuous at sites of aberrant budding. An increase in collagen and extensive infiltration of macrophages, which have recently been implicated in branching morphogenesis, is observed in the stroma surrounding the p190-B overexpressing TEBs. We propose that the stromal response, disruption of the myoepithelial layer, and alterations in IGF signaling in the p190-B overexpressing mice impact the TEB architecture leading to disorganization and increased branching of the ductal tree. Moreover, we suggest that alterations in tissue architecture and the adjacent stroma as a consequence of p190-B overexpression during pregnancy leads to loss of growth control and the formation of hyperplasia. These data demonstrate that precise control of p190-B RhoGAP activity is critical for normal branching morphogenesis during mammary gland development.

Introduction

Mammary gland ductal morphogenesis is a complex developmental process during which mammary epithelial cells must proliferate, migrate into the stromal fat pad, and differentiate into luminal and myoepithelial cell compartments (1). These processes occur within terminal end buds (TEBs), which drive the ductal penetration into the stromal fat pad (2). Ductal outgrowth is particularly dependent upon stromal-epithelial interactions, which provide proliferative and apoptotic cues as well as signals that effect cell migration (3, 4). Through their interactions with integrins, Rho GTP-binding proteins function to integrate extracellular signals to ultimately affect cell movement, proliferation, survival, and differentiation all of which are essential events during ductal morphogenesis (5).

Precise regulation of Rho GTPase activity is critically important, and several families of proteins including the Rho GTPase activating proteins (GAPs) are capable of modulating their activity (6). RhoGAPs function as negative regulators of Rho activity by enhancing the intrinsic GTPase activity of the Rho proteins to rapidly convert active GTP-bound Rho to inactive GDP-bound Rho (7). The role of the Rho signaling pathway in mammary gland development and breast cancer progression is not well understood. Several studies have reported overexpression of Rho family members in human breast cancers (8-10), and a number of reports have delineated functions of the Rho pathway by introducing dominant negative or active forms of Rho into breast cancer cell lines (11).

Until recently, Rho signaling in normal mammary gland development had not been examined. P190-B is a Rho specific RhoGAP (12) that is expressed in TEBs, and homozygous deletion of this RhoGAP gene completely inhibits ductal outgrowth (13, 14). Loss of one allele

of p190-B results in decreased proliferation within the TEBs causing a transient delay in ductal morphogenesis. Thus, mammary gland development is critically dependent on p190-B RhoGAP.

To further elucidate the role of p190-B in mammary gland development and tumor progression, a tetracycline (tet)-regulatable p190-B overexpressing mouse model was developed. This inducible system was chosen because it allows for manipulation of p190-B expression during distinct stages of mammary gland development and function. Using this approach, p190-B overexpression during ductal morphogenesis is shown to drastically alter TEB architecture. As a result, ductal elongation is delayed, branching is increased, and organization of the ductal tree is disrupted. Overexpression of p190-B during pregnancy results in hyperplastic lesions, which persist after postlactational involution. These studies demonstrate for the first time that overexpression of a RhoGAP is sufficient to disrupt mammary gland architecture and promote hyperplasia, confirming our previous findings that precise regulation of p190-B RhoGAP is critically important in the developing mammary gland.

Results

P190-B overexpression results in aberrant terminal end bud architecture

To generate the tet-regulatable p190-B overexpressing mice a transgene construct was designed in which the human p190-B cDNA was subcloned into TMILA containing the tetracycline operator/minimal CMV promoter elements followed by an inter-ribosomal entry site (IRES)-luciferase (Figure 1) (15). The presence of the IRES-luciferase allows for rapid identification of transgene expression in the mammary gland following induction. Injection of this construct into the pro-nuclei of fertilized FVB oocytes yielded nine founder lines as determined by Southern blot and PCR analyses (Figure 1 and data not shown).

To identify lines containing inducible p190-B transgene expression bigenic mice were obtained by breeding the p190-B founder mice to MMTV-rtTA (MTB) mice that express the reverse tetracycline transactivator (rtTA) under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) (Figure 1A) (16). Five week-old bigenic female mice from each line were treated with the tetracycline analog doxycycline (Dox) at 2 mg/ml in their drinking water for 3-7 days to induce transgene expression. Dox-treated MTB mice served as controls. Following treatment, mammary glands were dissected from the mice and analyzed for luciferase activity, p190-B transgene expression by RT-PCR, and morphological changes by whole-mount mammary gland analysis (Figure 1C). Three of the lines (6667, 6671, and 6674, denoted with asterisks in Figure 1B) showed inducible p190-B transgene expression as determined by luciferase activity and RT-PCR (Figures 1D-E).

Strikingly, within 3 days of p190-B transgene induction aberrant terminal end buds (TEBs) with extensive budding off the neck region of the TEBs were apparent in the whole-mounted mammary glands (Figure 1C). Histological analysis of hematoxylin and eosin (H&E) stained tissue sections from these glands further demonstrated the extent of disruption of the TEB architecture (Figures 2A-B). The TEBs exhibited extensive budding, abnormal morphologies, and disorganized and thickened stroma that in some cases encompassed the leading edge of the TEB. To quantify the extent of disruption of the TEB structures in the p190-B overexpressing mice, the percentage of normal TEBs was determined following 3 days of transgene induction. Structures were designated normal if they did not exhibit budding off the neck region of the TEB. In comparison to the Dox-treated control mice (n=6, 57 TEBs analyzed), the percentage of normal TEBs was significantly decreased in the p190-B overexpressing mice (n=5, 67 TEBs analyzed), 98.2+/-1.85 vs. 34.8+/-12.2 ($p<.0003$),

respectively (Figure 2E). Despite the pronounced TEB anomalies seen after short-term p190-B overexpression, the mature ducts in these mice did not exhibit any overt morphological abnormalities (Figures 2C-D). These phenotypes were not observed in the dox-treated MTB control mice. All three inducible lines showed the TEB phenotype, and subsequent studies were performed on two of the lines 6667 and 6671.

P190-B overexpression is predicted to alter signaling downstream of Rho. One of the immediate downstream targets of the Rho signaling pathway is Rho kinase (ROK). An increase in p190-B activity is expected to inhibit Rho activity, and consequently, decrease ROK activity. To examine whether this is the case, Western blotting for the phosphorylated active form of ROKII (pROKII/Thr396) was performed. In comparison to the MTB control, pROKII expression was substantially diminished in the p190-B overexpressing mammary glands as compared to the MTB control glands (Figure 2F). Total ROK expression, however, was equivalent between the MTB and p190-B overexpressing mice. This result demonstrates that overexpression of p190-B in the mammary gland inhibits signaling downstream of Rho.

To assess whether the newly formed buds off the neck region of the TEBs will persist or regress cell proliferation and apoptosis were evaluated. To detect proliferation within the buds immunohistochemical staining for the proliferation marker Ki67 was performed. As seen in Figure 3B, the aberrant budding structures extending from the TEBs in the p190-B overexpressing mice are highly proliferative. Apoptosis was assessed by immunohistochemical staining for the apoptotic marker cleaved caspase-3, and few cells within the aberrant buds are undergoing apoptosis (Figure 3D). These data suggest that the newly formed buds will grow out to form branches since the cells within the buds are proliferating and undergoing apoptosis similarly to cells within the control TEBs. Taken together, these data demonstrate that short-

term p190-B overexpression disrupts TEB morphology and may lead to aberrant branching off of the neck of the TEB.

To further examine the morphological abnormalities seen in the p190-B overexpressing TEBs, immunohistochemical staining was performed for cap/myoepithelial and body cell markers, p63 and E-cadherin, respectively (Figures 3E-H). This analysis demonstrated that both cell types are present within the aberrant TEBs. However, p63 immunostaining revealed that the cap/myoepithelial cell layer surrounding the aberrant TEBs is discontinuous as compared to control TEBs (Figures 3G-H). Immunostaining for the myoepithelial marker smooth muscle actin also show discontinuity in this cell layer (data not shown). This analysis suggests that p190-B overexpression alters cell-cell or cell-ECM interactions to impact the myoepithelial cell layer surrounding the TEBs.

P190-B overexpression results in abnormal stroma surrounding the TEBs

Histological analysis of the H&E stained TEBs demonstrated that the stroma surrounding the TEBs was altered in the p190-B overexpressing mice. The stroma in the p190-B overexpressing glands appeared disorganized, thicker, and more cellular. The degree of stromal disorganization correlated with the extent of TEB disruption such that the TEBs with drastically altered morphologies had more pronounced stromal anomalies. To further examine the stromal changes occurring in the p190-B overexpressing mice Masson's trichrome staining was performed as it allows for visualization of aniline blue stained collagen fibers. As seen in Figure 4B, the stroma surrounding the aberrant TEB from the p190-B overexpressing mammary gland is highly enriched in collagen fibers as compared the control TEB in which the collagen fibers are

localized primarily to the neck region of the TEB (Figure 4A). This result suggests that p190-B overexpression results in altered stromal-epithelial interactions during TEB outgrowth.

Immune cells, in particular macrophages and eosinophils, have recently been shown to play an important role in ductal morphogenesis in the developing mammary gland (17). Macrophages are also known to be involved in activation of stromal fibroblasts to affect extracellular matrix (ECM) deposition (18). The aberrant budding off the TEBs as well as the alterations in the stromal thickness and collagen deposition that were observed in the p190-B overexpressing mice suggested that there may also be alterations in immune cell infiltration surrounding the abnormal TEBs. To examine this possibility, immunohistochemical staining for the macrophage and eosinophil marker F4/80 was performed. In contrast to the control TEBs, which have fewer F4/80 positive cells localized predominantly to the neck region of the TEBs, the number of cells staining positive for F4/80 in the stroma surrounding the aberrant TEBs is markedly increased (Figures 4C-D). Taken together, these data suggest that p190-B overexpression influences immune cell infiltration within the stroma adjacent to the TEBs, which ultimately affects TEB architecture.

Downstream of p190-B RhoGAP are the insulin receptor substrate (IRS) proteins 1 and 2 (19). Deficiency of p190-B leads to increased Rho kinase (ROK) activity and phosphorylation of the IRS proteins, which targets them for degradation. As a result, insulin like growth factor receptor (IGFR) signaling is diminished. Signaling through the IGFR pathway has also been shown to play an important role in mammary gland ductal morphogenesis since IGF-IR deficiency impairs take rate and ductal outgrowth in mammary gland transplantation studies (20). Constitutive activation of IGF-IR increases ductal side branching, delays ductal outgrowth, and results in rapid formation of adenocarcinomas (21). To determine whether signaling

through the IGFR pathway is altered in the aberrant TEBs in the p190-B overexpressing mice immunohistochemistry for IRS-1, IRS-2, and a downstream target of the IGFR signaling pathway, phospho-Akt, was performed. Interestingly, this analysis revealed a reduction in IRS-1 and IRS-2 expression levels (Figures 5A-D) as well as a reduction in phospho-Akt in the aberrant TEBs as compared to control TEBs (Figures 5E-F). While a decrease in expression of these proteins was not predicted, these results indicate that p190-B overexpression is impacting signaling through the IGFR pathway.

Long-term p190-B overexpression during virgin mammary gland development results in disorganization of the ductal tree

To investigate the consequences of long-term overexpression of p190-B on ductal morphogenesis, bigenic (n=6, 3 mice from each line) and wildtype (n=4) littermate control mice were treated continuously with Dox beginning at 5.5 weeks of age until 9.5 weeks of age at which time the growing ducts normally reach the end of the fat pad. In comparison to the Dox-treated wildtype littermate control mammary glands, which showed normal architecture within the ductal tree, long-term p190-B overexpression resulted in disorganization of the ductal tree and increased branching as seen in the whole-mounted mammary glands (Figure 6B). Luciferase assays and RT-PCR were performed to confirm expression of the p190-B transgene (Figure 6E and data not shown). To quantify the increase in ductal branching the average number of secondary and tertiary branch points was compared between the control and p190-B overexpressing mice. This analysis revealed a significant increase in branching in the p190-B overexpressing mice (21.9+/-1.84 vs. 15.5+/-1.93, $p<.03$) as compared to the control mice (Figure 6F). Histological examination of H&E stained tissues sections demonstrated that long-

term p190-B overexpression throughout ductal morphogenesis results in the presence of abnormal TEBs and thickened stroma surrounding the mature ducts (Figure 6C-D). Normally at 9.5 weeks of age, the TEBs have reached the end of the fat pad and begin to regress. TEBs were still detected in the most disorganized p190-B overexpressing mammary glands in which the ducts had not yet reached the end of the fat pad. Proliferation rates within the mature ducts were similar between the p190-B overexpressing and Dox-treated wildtype littermate control mice as determined by quantification of Ki67 positive cells (2.6 +/- 0.97 vs. 2.1+/-1.1, p>.07). Taken together, these data demonstrate that the aberrant budding off the TEBs in the p190-B overexpressing mice results in a disorganized ductal tree with increased branching and altered stroma surrounding the mature ducts.

Upon examination of the long-term p190-B overexpressing mammary glands it was noted that the severity of the aberrant branching and disorganization within the growing ductal tree correlated with a delay in ductal outgrowth. To further investigate the affects of p190-B overexpression on ductal elongation, p190-B overexpressing mammary tissue was transplanted into the cleared fat pads of three week-old female mice. As a control, MTB tissue was transplanted into the contralateral cleared number four fat pads. The transplants were allowed to grow out for eight weeks at which time the mice were bred to wildtype FVB males. Three days after parturition mammary glands were collected and analyzed by H&E staining. Luciferase assays were performed to confirm transgene expression (data not shown). Interestingly, p190-B overexpression resulted in a dramatic delay in ductal outgrowth as compared to the MTB controls. This analysis revealed that 100% (6/6) of the MTB control transplants completely filled the fat pad whereas only 33% (2/6) of the p190-B overexpressing transplants filled the fat pad (Figure 6G). The remaining four p190-B overexpressing transplants filled the fat pad 50%

or less (data not shown). These data demonstrate that p190-B overexpression delays ductal morphogenesis, and pregnancy does not rescue this defect.

Overexpression of p190-B during pregnancy results in hyperplastic lesions

To examine the affects of p190-B overexpression during pregnancy and lactation, 12 week-old bigenic (n=6), and MTB (n=3) and wildtype littermate (n=3) control mice were bred to wildtype male mice. To induce p190-B transgene expression dox-treatment was started when the males were placed with the females and continued throughout pregnancy and lactation. MTB and wildtype control mice were also Dox-treated. During late pregnancy (days 16-18) millimeter-sized biopsy samples were collected from the bigenic (n=2) and wildtype littermate (n=2) control mice. Interestingly, histological examination of H&E stained sections of biopsy samples from both bigenic mice showed hyperplastic lesions that were readily detectable within the small samples that were collected (Figure 7C-D). Neither of the wildtype controls contained hyperplastic lesions. Furthermore, hyperplastic lesions were detected in involuted mammary glands from the p190-B overexpressing mice, but not in the MTB controls (Figure 7B). Overexpression of p190-B, however, did not inhibit lactation since all six bigenic mice were able to support their litters (6 or more pups) to weaning age.

Discussion

Reported here for the first time is an *in vivo* model in which the effects of tet-regulatable p190-B RhoGAP overexpression on mammary gland development and function are examined. To date, investigation of the role of the Rho pathway in the mammary gland have been performed primarily in breast cancer cell lines in which Rho signaling is manipulated by

overexpression of either dominant active or inhibitory forms of Rho (11). More recently, siRNA has been used to downregulate specific Rho family members in breast cancer cell lines (22, 23). These studies have elucidated roles for the Rho signaling pathway in proliferation, adhesion, and invasion of breast cancer cells. However, they have not allowed for examination of this pathway in normal mammary epithelial cells in the context of the *in vivo* environment, which includes stromal-epithelial interactions that are critical for mammary gland development, function, and breast cancer progression. In the current study, the p190-B transgene is under the control of a tet-regulatable promoter, which provides temporal control allowing for investigation of the effects of overexpression of this RhoGAP at distinct stages of mammary gland development and function. This unique aspect allowed for the effects of p190-B overexpression on ductal outgrowth in the developing virgin and during pregnancy and lactation to be examined.

Short-term overexpression of p190-B during ductal morphogenesis dramatically altered the architecture of the TEBs and the adjacent microenvironment. The abnormal TEBs were characterized by extensive budding off the neck region, disruption of the myoepithelial cell layer, and pronounced stromal alterations. Overexpression of p190-B throughout ductal morphogenesis resulted in delayed ductal elongation, disorganization of the ductal tree, and increased side branching. Previously, loss of p190-B was shown to completely inhibit ductal morphogenesis (14). Haploinsufficiency of p190-B was shown to transiently delay ductal morphogenesis, due to decreased proliferation in the cap cell layer of the TEB possibly resulting from diminished expression of IRS proteins (14). The current study complements the loss of function studies and demonstrates that precise regulation of p190-B in the developing mammary gland is required for normal TEB structure, ductal elongation, and organization of the ductal tree.

P190-B overexpression resulted in several cellular and molecular changes within the TEBs and surrounding microenvironment, all of which are likely to contribute to the aberrant TEB architecture. Interestingly, within the abnormal TEBs discontinuity of the myoepithelial cell layer was detected at sites of aberrant budding. Myoepithelial cells, which secrete a number of proteases, contribute to maintenance and remodeling of the extracellular matrix underlying the ductal epithelium (24). Furthermore, myoepithelial cells may have tumor suppressor roles since they have been shown to inhibit proliferation, induce apoptosis, and block invasion of breast cancer cells (25-27). Mice overexpressing an inducible form of fibroblast growth factor receptor 1 (MMTV-iFGFR1) also had a noncontiguous myoepithelial cell layer at sites of aberrant branching (28). Thus, disruption of the myoepithelial cell layer in the p190-B overexpressing TEBs may play an important role in the aberrant budding off the neck region of the TEBs. The molecular mechanisms by which overexpression of p190-B contributes to alterations in the myoepithelial cell layer remain unclear. Future studies examining the interactions between primary myoepithelial and luminal epithelial cells isolated from the tet-regulatable p190-B overexpressing mice in a three-dimensional culture system will help to elucidate the molecular signaling pathways involved in the cross-talk between the myoepithelial and luminal epithelial cells.

Another phenotype observed in the aberrant TEBs was a pronounced alteration in the adjacent microenvironment. The stroma was thicker, more cellular, and contained more collagen as determined by Masson's trichrome staining. Recently, elegant studies by Dr. Valerie Weaver and colleagues demonstrated that matrix rigidity plays a critical role in epithelial morphogenesis (29). Rho-dependent cytoskeletal tension is increased in epithelial cells grown on a stiff stroma altering cell-cell/cell-matrix adhesion and polarity to ultimately disrupt morphogenesis. These

studies demonstrated that even small increases in matrix stiffness are sufficient to increase cell growth and compromise tissue architecture. Furthermore, ROCK-mediated contractility is required for breast epithelial cells to sense the rigidity of their environment, and downregulation of Rho activity is necessary for epithelial cell differentiation (30). Thus, the increase in collagen and stromal thickness adjacent to the aberrant TEBs may result in a more rigid stroma leading to disrupted TEB architecture in the p190-B overexpressing mice.

Interestingly, ROK activity is decreased in the p190-B overexpressing mammary glands. The Rho pathway plays an essential role in regulation of actin cytoskeletal dynamics, and remodeling of the actin cytoskeleton is required for a number of cellular processes (31). Continual inhibition of Rho signaling by overexpression of p190-B RhoGAP, therefore, is not likely to be tolerated within normal mammary epithelial cells. Thus, it is probable that a compensatory upregulation of other signaling pathways that contribute to cytoskeletal regulation occurs in response to p190-B overexpression. The stromal response in the p190-B overexpressing mice may occur to compensate for the chronically depressed ROK activity allowing for modulation of Rho-dependent cytoskeletal tension.

In addition to the changes in matrix deposition, a significant increase in the number of immune cells was detected by immunostaining for the macrophage and eosinophil marker F4/80. While disruption of the myoepithelial cell layer may contribute to alterations in the stromal environment as discussed above, it is likely that p190-B overexpression modulates inside-out signaling pathways that influence immune cell infiltration and extracellular matrix deposition. Macrophages and eosinophils have been shown to play an essential role in branching morphogenesis of the mammary gland since depletion of these cells inhibited ductal branching and elongation (17). Their ability to promote ECM remodeling and aid in the release of growth

factors may contribute to the disruption in ductal morphogenesis seen in the p190-B overexpressing mice. Thus, the aberrant budding off the TEBs and increased branching observed after long-term p190-B overexpression is likely to be influenced by the marked increase in F4/80 positive immune cells observed in association with the aberrant TEBs. The molecular mechanisms by which overexpression of p190-B contributes to the recruitment of immune cells remain unclear. Recently, in breast cancer cells the Rho signaling pathway was shown to be important for production of colony stimulating factor 1 (CSF1) (32), which is a major regulator of macrophage activation (33). Thus, it is possible that p190-B overexpression leads to alterations in Rho signaling that impact expression of CSF1 expression leading to the recruitment of macrophages.

Similar to p190-B RhoGAP, IGF-IR is critical for normal mammary gland ductal morphogenesis. In transplant studies, embryonic mammary buds deficient for IGF-IR show a significant reduction in take rate and ductal outgrowth is severely impaired (20). Overexpression of a constitutively active IGF-IR in the developing mammary gland increased side branching, delayed ductal elongation, and resulted in the rapid formation of adenocarcinomas (21). P190-B RhoGAP was recently shown to interact with the IGF signaling axis *in vivo*. Deficiency of p190-B resulted in increased the activity of ROK, which phosphorylates the IRS proteins targeting them for degradation (19). In addition, IGF-IR activation positively regulates p190-B activity through phosphorylation events that alter the subcellular location of p190-B (34). Furthermore, decreased expression of IRS-1 and IRS-2 was detected in the TEBs of p190-B heterozygous mice (14). Thus, interactions between IGF-IR and p190-B signaling are likely to play an important role in the developing mammary gland.

In the current report, expression of the IRS proteins and activation of the downstream effector AKT was significantly reduced in the aberrant TEBs. This result was initially surprising as it was predicted that p190-B overexpression would lead to stabilization of the IRS proteins and increased activation of downstream signaling. Expression of these proteins was examined after 3-7 days of p190-B transgene induction. Initially there may be increased IGFR signaling in response to p190-B overexpression, which contributes to the aberrant budding off the neck of the TEB. Once the branching is initiated, p190-B overexpression may invoke negative feedback regulation leading to decreased IRS protein levels and phospho-AKT. This is not unprecedented, as it has been shown that after sustained IGFR stimulation IRS proteins are targeted for degradation through the phoshpatidylinositol 3'-kinase/Akt/mTOR pathway (35). Sustained activation of the IGF-IR pathway has indeed been shown to induce aberrant branching and delay ductal elongation as discussed above (21). Thus, it is attractive to speculate that alterations in IGF signaling downstream of p190-B are involved the abnormal budding, increased branching, and delayed ductal elongation that was detected in the p190-B overexpressing mice. It is possible that altered IGF signaling is not a direct consequence of p190-B overexpression, however, this remains to be determined.

The ability to temporally regulate expression of the p190-B transgene allowed for examination of the role of p190-B overexpression during distinct stages of mammary gland development and function. To investigate the consequences of p190-B overexpression during pregnancy and lactation, the transgene was induced on the first day of pregnancy and continued throughout lactation. P190-B overexpression did not have any apparent effect on lactation. Interestingly, hyperplastic lesions were detected in biopsies from p190-B overexpressing pregnant and involuted mammary glands. To our knowledge, this is the first report in which

overexpression of a RhoGAP was shown to have neoplastic activities *in vivo*. One potential explanation for the development of these lesions is that there may also be perturbations in the myoepithelial cell layer when p190-B is overexpressed during pregnancy, which could lead to a loss of growth control as discussed above. Another potential explanation is that alterations in the IGF-IR signaling axis, which has been shown to result in the formation of adenocarcinomas *in vivo,* may occur in response to p190-B overexpression. Alternatively, increased rigidity of the stroma may lead to loss of growth control and tissue architecture (29). Analysis of multiparous mice in which p190-B is chronically overexpressed is necessary to determine whether these hyperplastic lesions will progress. This study as well as transplantion of the hyperplastic lesions is currently ongoing.

Materials and Methods

Transgenic mice

To generate the tetracycline regulatable p190-B transgenic mice the following construct was engineered. The 4.9 kb human p190-B cDNA was subcloned into the TMILA tetracycline operator (TetO)-IRES-luciferase vector downstream of the TetO (15). A 7.2 kb fragment containing the TetO-p190-B-IRES-luciferase expression cassette was microinjected into the pronuclei of fertilized FVB/N oocytes by the Baylor College of Medicine Transgenic Mouse Core yielding nine potential founder lines. Southern blotting to detect the transgene in genomic DNA prepared from tail cuts was performed to identify founder lines. Mice were maintained on an inbred FVB/N background.

Bigenic mice were obtained by breeding TetO-p190-B-IRES-luciferase mice to MTB mice, which contain the reverse tetracycline transactivator under the control of the MMTV promoter (16). For genotyping, PCR amplification of the MTB and p190-B transgenes was done on genomic DNA prepared from tail cuts using the following oligonucleotide pairs: for TetO-p190-B, 5'-CCTCAAAAAAGTCATGGGGAACGGAGC-3' and 5'-CGCTGACACGGTAGAGTCCTTCGG-3'; for MTB, 5'-TCCAAGGGCATCGGTAAACA-3' and 5'-GCATCAAGTCGCTAAAGAAG-3'. Reaction conditions were 94° for 3 min followed by 30 cycles of 94° for 30 sec, 60° for 45 sec, 72° for 45 sec, followed by a 5 min extension at 72°. To induce transgene expression bigenic mice were treated with doxycycline (Dox) (Clontech, Mountain View CA) at 2 mg/ml in their drinking water containing 5% sucrose. Fresh Dox water was given twice weekly. Animal care and procedures were approved by the Institutional Animal Care and Use committee at Baylor College of Medicine and were in

accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (NIH publication 85-23).

For mammary gland transplantation the inguinal #4 mammary glands from 21 day-old FVB/N female mice were cleared of the mammary epithelium as previously described (36). Small pieces of tissue ~1mm^3 in size were transplanted into the cleared fat pads. Transplants were allowed to grow out for 8 weeks. Mammary gland tissue isolation and whole-mount preparation was performed as previously described (37). Analysis of branch points and TEB morphology was performed blinded by examining whole-mounted mammary glands. For branch point analysis the primary duct was identified starting at the nipple, and the average number of secondary and tertiary branch points off the primary duct was determined. The unpaired Student's t-test was used to determine statistical significance.

Luciferase assay

Snap-frozen mammary tissue was ground using a mortar and pestle. Tissue extracts were prepared in Passive Lysis Buffer (Promega, Madison WI), and cleared by centrifugation. Luciferase activity was measured using Promega's Luciferase Assay System according to the manufacturer's instructions. Protein concentrations in the tissue extracts were determined using the BCA Protein Quantitation Assay (Pierce, Rockford IL).

Southern hybridization

A random primed (DNA labeling kit, Roche, Indianapolis IN) cDNA probe recognizing the first 1.3 kb of the human p190-B coding region was used to probe Southern blots containing

EcoRI-digested genomic DNA prepared from tail cuts as previously described (38). The digested DNA was transferred to Zetaprobe (BioRad Hercules, CA).

Immunohistochemical staining

Paraffin embedded tissue sections (5 μm) were deparaffinized in xylenes, and rehydrated through a series of graded ethanols. Tissue sections were then stained with hematoxylin and eosin, Accustain (Masson's) Trichrome stain (Sigma-Aldrich, St. Louis MO), or antibodies to detect specific proteins. Antigen retrieval was performed by microwaving slides in 10 mM citrate, pH 6, for 20 min. For immunostaining with mouse monoclonal antibodies, the M.O.M kit (Vector, Burlingame CA) was used to block non-specific binding and for dilution of primary antibodies. For primary polyclonal antibodies, the tissue sections were blocked and primary antibodies were diluted in a 5% solution of bovine serum albumin in PBS + 0.5% Tween-20. Sections were incubated with primary antibody overnight at room temperature. The following antibodies and dilutions were used: E-cadherin 1:250 (BD Transduction Labs, San Jose CA), Ki67 1:5,000 (Santa Cruz Biotechnology, Santa Cruz CA), cleaved caspase-3 (Asp175) 1:1000 (Cell Signaling, Beverly MA), p63 1:500 (Lab Vision Neomarkers, Fremont CA), IRS-1 1:800 (Upstate Biotech, Waltham MA), IRS-2 1:800 (Upstate Biotech), pAKT (Ser473) 1:50 (Cell Signaling), F4/80 1:50 with no antigen retrieval (Caltag Laboratories, Burlingame CA). Biotinylated anti-rat (Molecular Probes, Carlsbad CA), anti-rabbit (Oncogene Research, Darmstadt Germany), and anti-mouse (Oncogene Research) secondary antibodies were diluted 1:200 in PBS and were incubated on the tissue sections for 1 hr at room temperature. Vectastain Elite ABC and diaminobenzidine (DAB) substrate kits were used to detect immunoperoxidase staining according to the manufacturer's instructions (Vector Laboratories, Burlingame CA).

RT-PCR

RNA was prepared from mammary glands using Trizol Reagent according to the manufacturer's recommendations. To prepare cDNA, 1μg of RNA was first DNase treated, primed with oligo-dT, and reverse transcribed using MMLV-reverse transcriptatse (all reagents for RT were purchased from Invitrogen, Carlsbad CA). The oligonucleotides that were used for genotyping PCR were also used for RT-PCR. As a negative control reactions were also performed in the absence of RT (data not shown). Amplification of L19 served as a control for the RT reaction. The following oligonucleotides and conditions were used: 5'-AGTATCCTCAGGCTTCAGAA-3' and 5'-TTCCTTGGTCTTAGACCTGC-3'. Reaction conditions were 94° for 3 min followed by 30 cycles of 94° for 30 sec, 60° for 45 sec, 72° for 45 sec, followed by a 5 min at 72°.

Western blotting

To examine expression and phosphorylation of ROKII mammary gland extracts were first prepared by pulverizing snap-frozen tissues followed by lysis in Passive Lysis Buffer (Promega) containing a protease inhibitor cocktail (Roche) and clearing by centrifugation. Protein concentrations in the tissue extracts were determined using the BCA Protein Quantitation Assay (Pierce). Mammary gland extracts were prepared from p190-B overexpressing (n=4) or MTB control glands (n=4) at day 3 of involution that had been treated continuously with Dox throughout pregnancy and involution. Extracts were pooled (20 μg of each), electrophoresed on 6% SDS-PAGE gels, and transferred to PVDF membrane (Millipore, Bedford MA). Membranes were blocked in 5% milk/TBS followed by incubation with phopho-ROKII (thr396) (AnaSpec Incorporated, San Jose CA) antibody diluted 1:1000 in 5% milk/TBST. Peroxidase conjugated

goat anti-rabbit secondary antibody (Jackson Immunologicals) was used at 1:5000, and signal was detected with Supersignal West Pico Solutions (Pierce, Rockford IL). The membrane was stripped and re-probed with anti-ROKII (AnaSpec) diluted 1:1000 to detect total ROKII. To control for loading, Western blotting for ERK was performed using and anti-ERK antibody (Cell Signaling) diluted 1:1000. Fast green stain was also used to confirm equal loading of proteins on the membranes (data not shown).

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Figure Legends

Figure 1: Generation of Tet-regulatable p190-B overexpressing mice. A. A schematic of the two constructs used to generate bigenic tet-regulatable p190-B overexpressing mice. B. Southern blot analysis of p190-B overexpressing founder lines. *Indicates inducible lines. C. Whole-mount mammary glands showing aberrant budding off the neck region of p190-B overexpressing TEBs compared to uninduced bigenic controls after 3 days of transgene induction. All three inducible lines showed this phenotype. Representative images are shown. D. Luciferase activity shown as relative light units (RLUs) per mg of protein in mammary glands from control and Dox-induced mice. E. RT-PCR analysis of p190-B transgene expression in mammary glands from control and Dox-induced mice. RT-PCR for L19 is shown as a control for the RT reaction. Reactions performed in the absence of reverse transcriptase did not contain products (data not shown).

Figure 2: P190-B overexpression disrupts TEB architecture. Representative images of H&E stained mammary gland tissue sections from bigenic or MTB control mice treated with Dox for 3 days are depicted. A. A TEB from control mammary gland with normal architecture is shown. B. A TEB from a p190-B overexpressing mammary gland with abnormal budding is shown (arrows). The mature ducts appear normal in the p190-B overexpressing mice (panel D) as compared to the MTB control mice (panel C). Scale bars represent 50 μm. E. The percentage of normal TEBs in p190-B overexpressing (Bigenic) compared to control (MTB) mammary glands is graphed. F. Western analysis of phosphorylated and total ROKII levels in p190-B overexpressing and control mammary glands. Total ERK is shown as a loading control.

Figure 3: Immunohistochemical analysis of proliferation, apoptosis, and cap/myoepithelial and body cells in p190-B overexpressing TEBs. A-B. Immunostaining for the proliferation marker ki67 shows abundant proliferation in the control TEBs as well as the aberrant buds (arrow) in the p190-B overexpressing TEBs. The inset shown in panel B demonstrates multiple buds extending from the TEB. C-D. Immunostaining for the apoptotic marker active caspase 3 shows that few cells are undergoing apoptosis in the abnormal buds similar to the control TEBs (arrows indicate positively stained cells). E-F. Immunostaining for E-cadherin marks the body cells in the TEBs. G-H. p63 immunostaining demonstrates non-contiguity of the myoepithelial cell layer in the p190-B overexpressing TEBs (arrows) as compared to the control TEB, which shows a continuous cap/myoepithelial cell layer. Note the thickened stroma surrounding the p190-B overexpressing TEBs. Scale bars equal 50 μm.

Figure 4: Decreased IGF receptor signaling in p190-B overexpressing TEBs. Immunohistochemical staining for IRS-1 (panels A and B), IRS-2 (panels C and D), and phospho-AKT (panels E and F) demonstrated that expression of the IRS proteins and phospho-AKT was significantly diminished in the aberrant p190-B overexpressing TEBS as compared to control TEBs. Scale bars equal 50 μm.

Figure 5: Abnormal stroma surrounds p190-B overexpressing TEBs. A-B. Masson's trichrome staining shows increased collagen deposition (arrows, blue color) in the stroma surrounding the aberrant p190-B overexpressing TEB as compared to the control TEB. Scale bars represent 100 μm. C-D. Immunostaining for the macrophage and eosinophil marker F4/80 shows abundant immune cells (arrows) within the stroma adjacent to the p190-B overexpressing TEB as

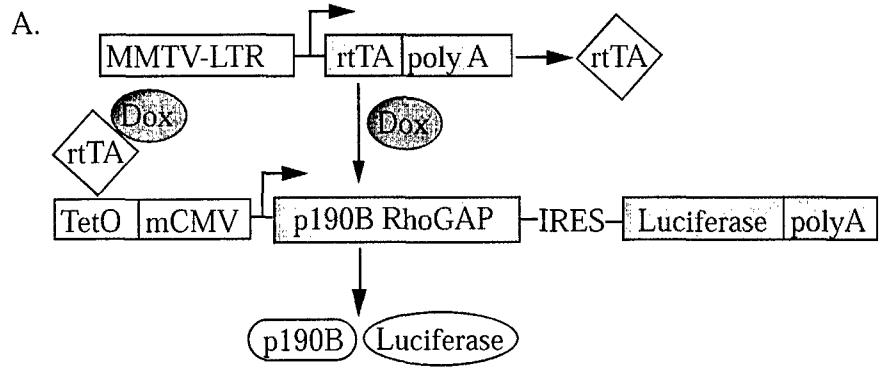
compared to the stroma surrounding the control TEB which contains fewer immune cells that are localized primarily to the neck region of the TEB. Scale bars represent 100 μm.

Figure 6: Persistent overexpression of p190-B during ductal morphogenesis results in delayed ductal elongation, increased branching, and disorganization of the ductal tree. A-B. Whole-mounted mammary glands from Dox treated wildtype control littermates and bigenic mice show aberrant architecture of the ductal tree in the p190-B overexpressing mammary gland as compared to the normal ductal tree seen in the Dox-treated control. Arrows indicate the presence of abnormal TEBs. C-D. H&E stained tissue sections show thickened stroma surrounding the ducts in the p190-B overexpressing mammary glands as compared to the thin layer of connective tissue surrounding the ducts in the control mammary glands. Scale bars equal 50 μm. E. RT-PCR shows p190-B transgene expression in the Dox-treated bigenic mice, but not in the wildtype controls. All wildtype controls were negative, and L19 and no RT controls were performed (data not shown). F. Quantification of side branching in long-term Dox-treated mammary glands revealed a significant increase in the number of side branches in the p190-B overexpressing mice compared to the Dox-treated wildtype control mice. The average number of branch points is graphed. G. The percentage of bigenic compared to MTB control transplants that filled the fat pad is graphed.

Figure 7: p190-B overexpression during pregnancy results in hyperplastic lesions. A-B. Hyperplastic lesions from p190-B overexpressing mice are shown in whole-mounted involuted mammary glands (arrow) (panel A) whereas no lesions were detected in the MTB control glands

(panel B). C-D. H&E stained sections showing the histology of the hyperplastic lesions from each biopsy. Scale bars represent 50 μm.

FIGURE 1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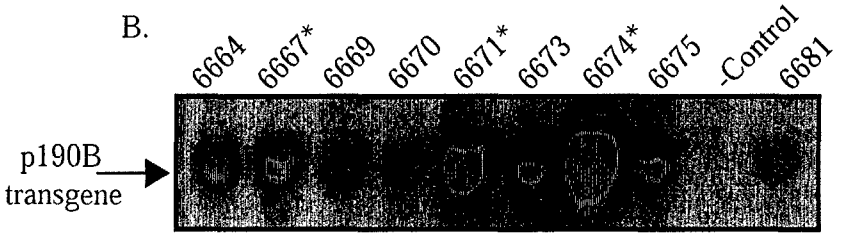
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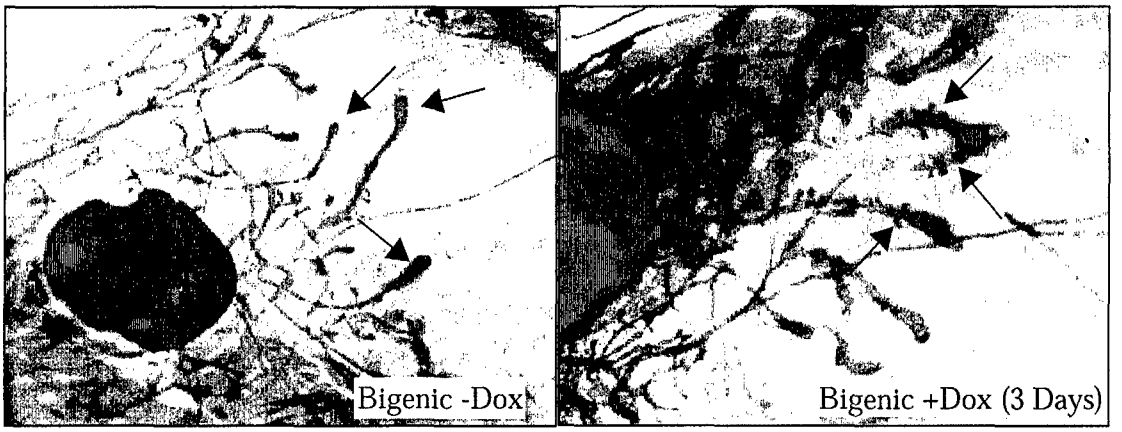
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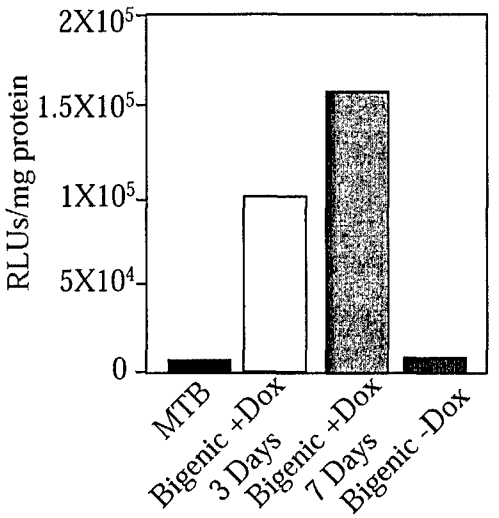
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Bigenic +Dox 7 Days
Bigenic -Dox

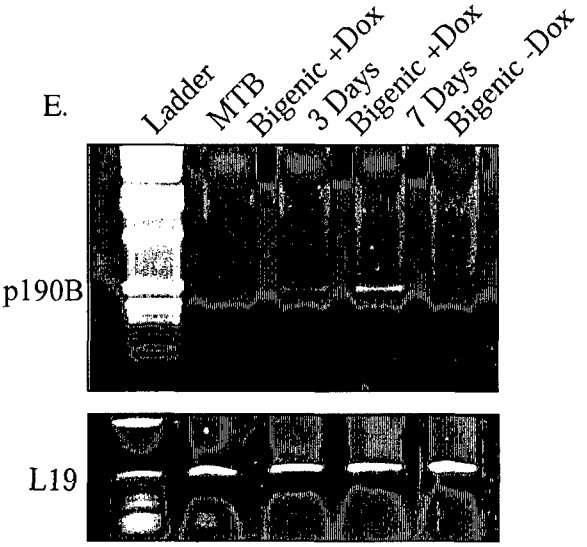
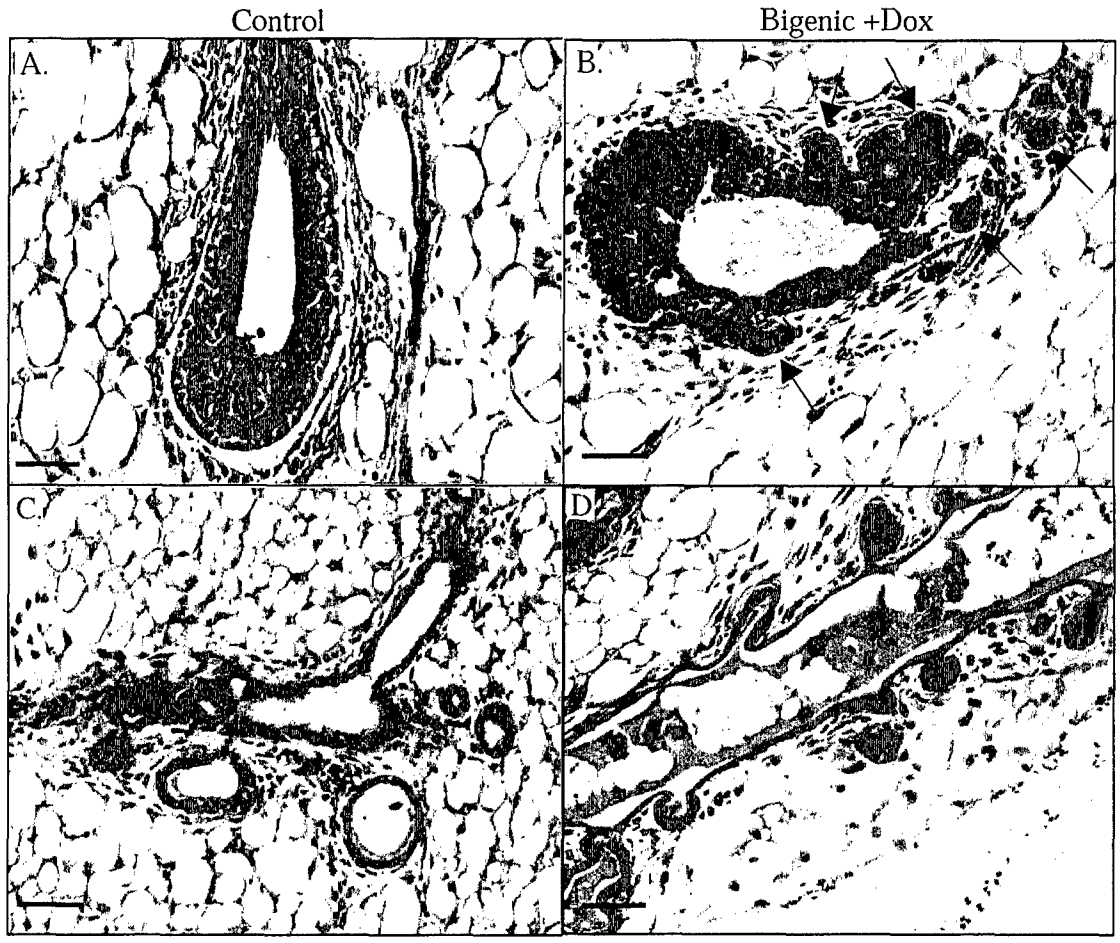
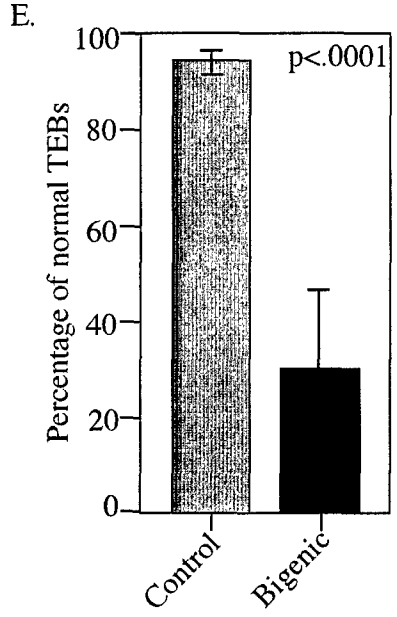

E.
Ladder
MTB
Bigenic +Dox 3 Days
Bigenic +Dox 7 Days
Bigenic -Dox
p190B
L19

FIGURE 2


Control
Bigenic +Dox
A.
B.
C.
D.


E.
100
80
60
40
20
0
p<.0001
Percentage of normal TEBs
Control
Bigenic

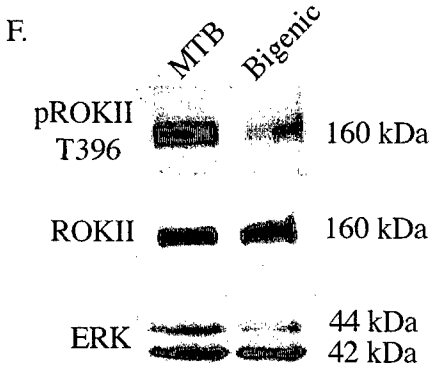

F.
MTB
Bigenic
pROKII
T396
160 kDa
ROKII
160 kDa
ERK
44 kDa
42 kDa

FIGURE 3

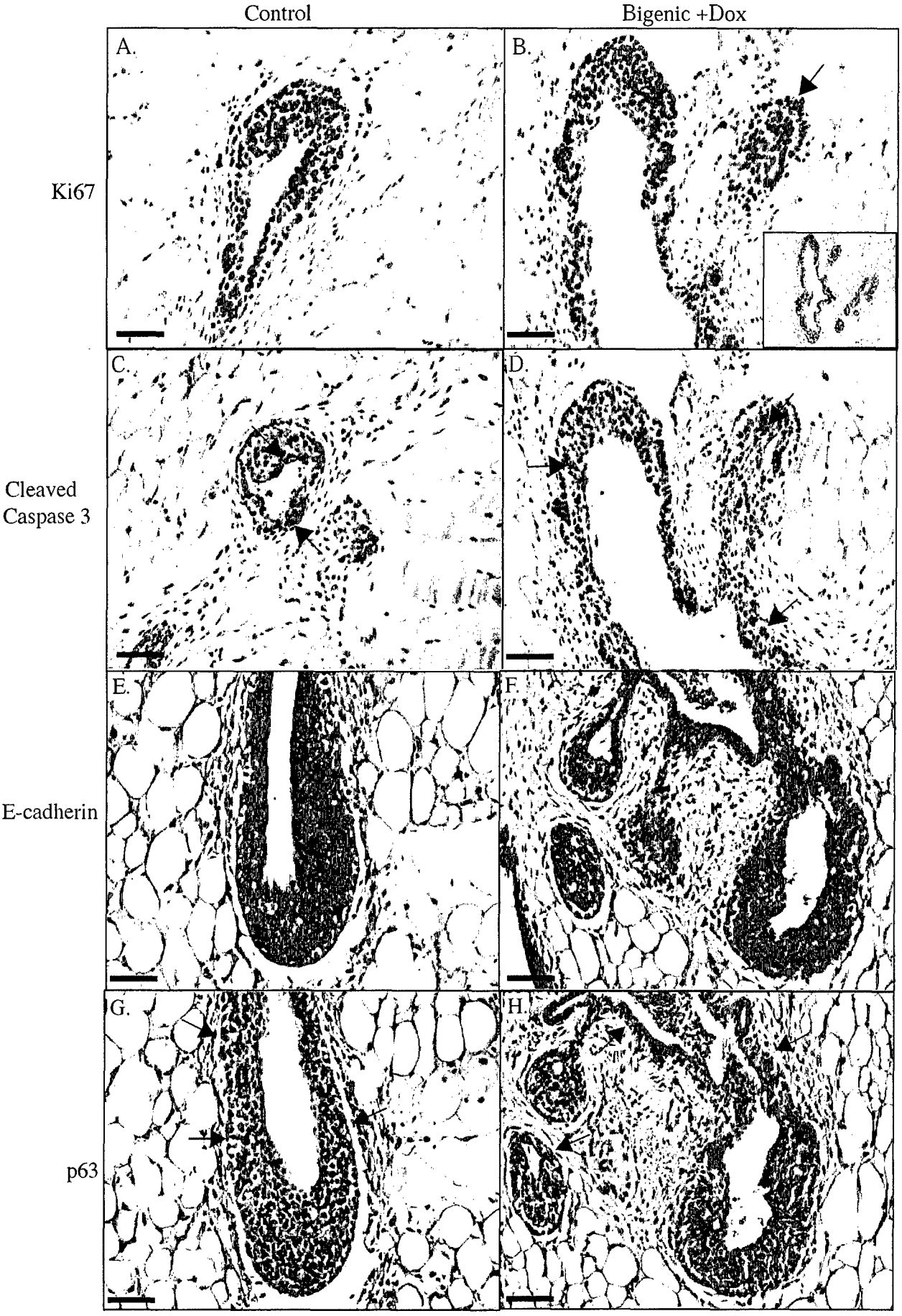

Control
Bigenic +Dox
A.
B.
Ki67
C.
D.
Cleaved
Caspase 3
E.
F.
E-cadherin
G.
H.
p63

FIGURE 4

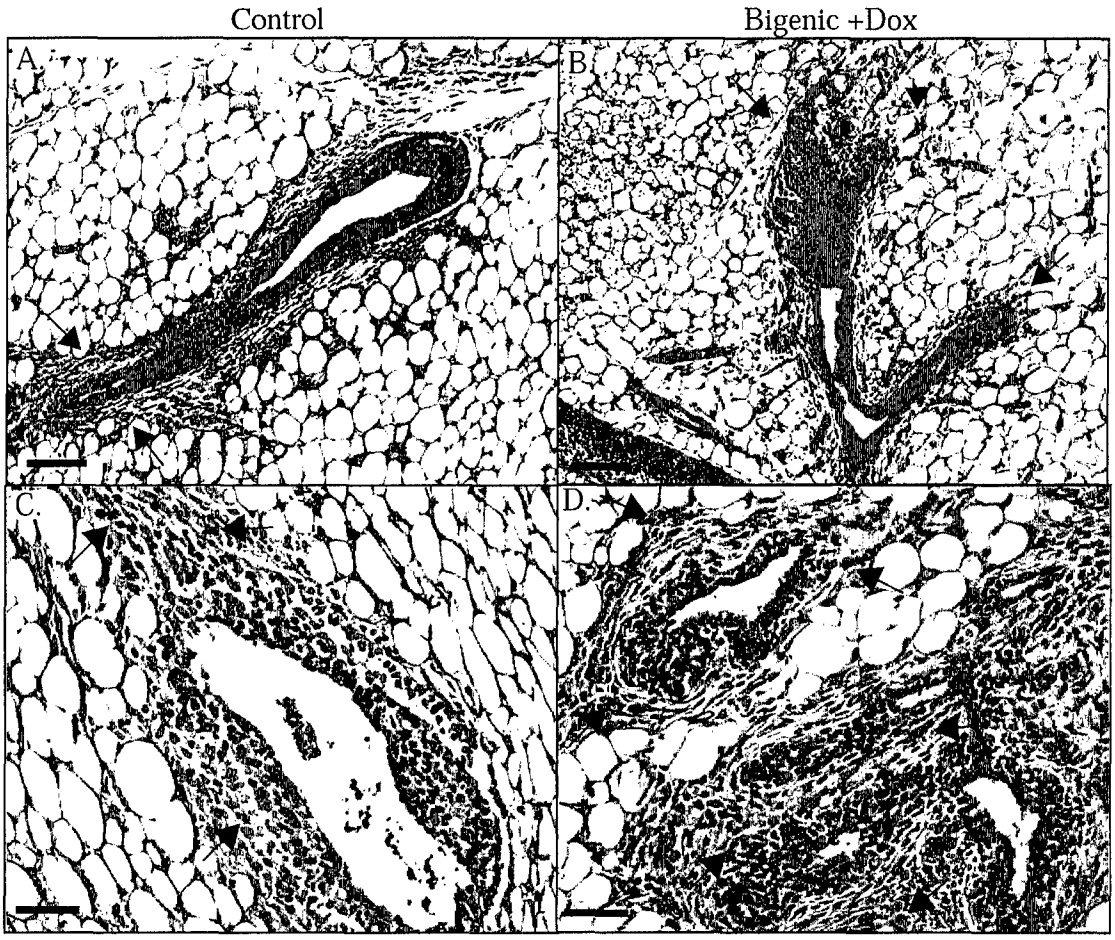


FIGURE 5

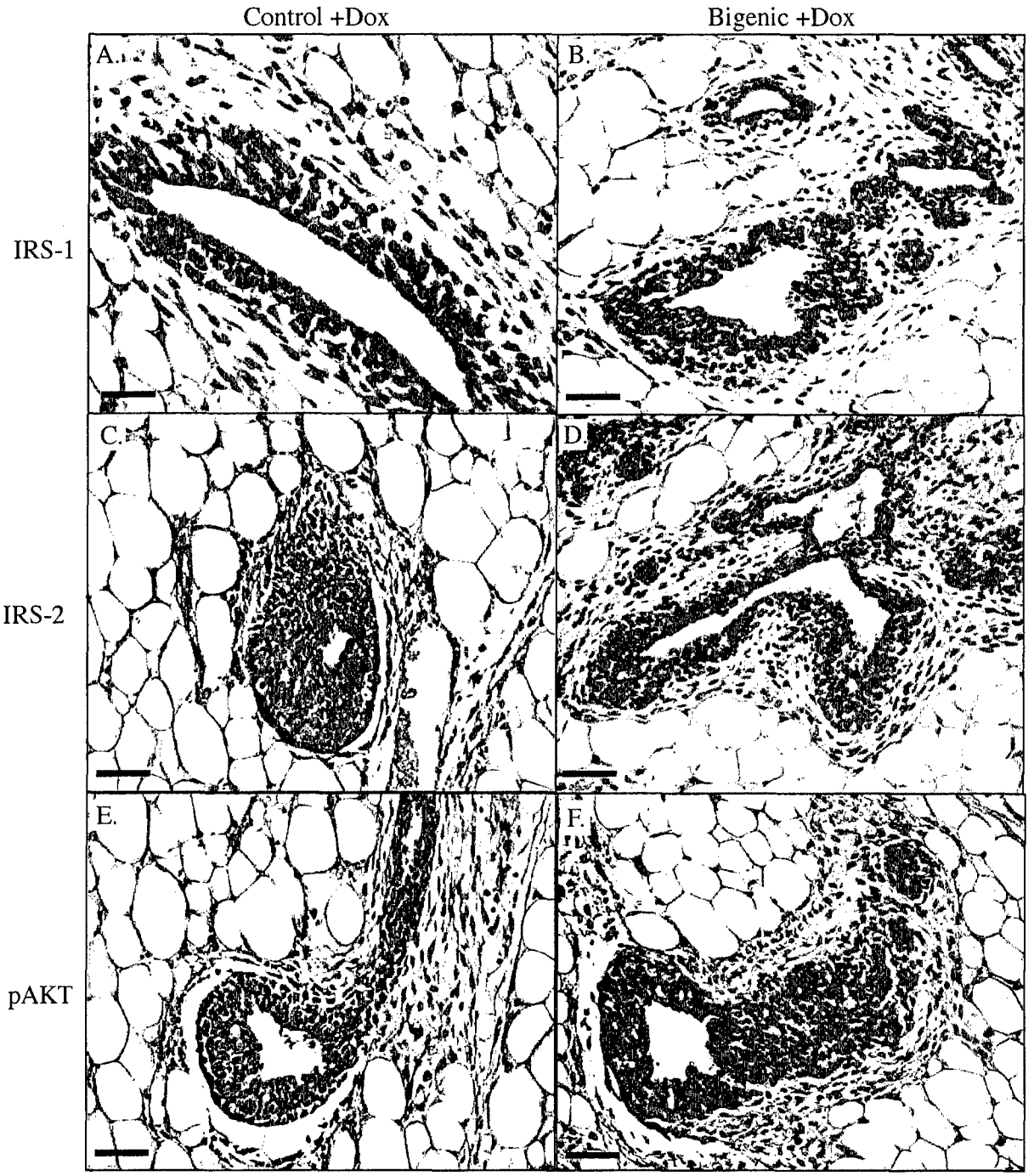


FIGURE 6

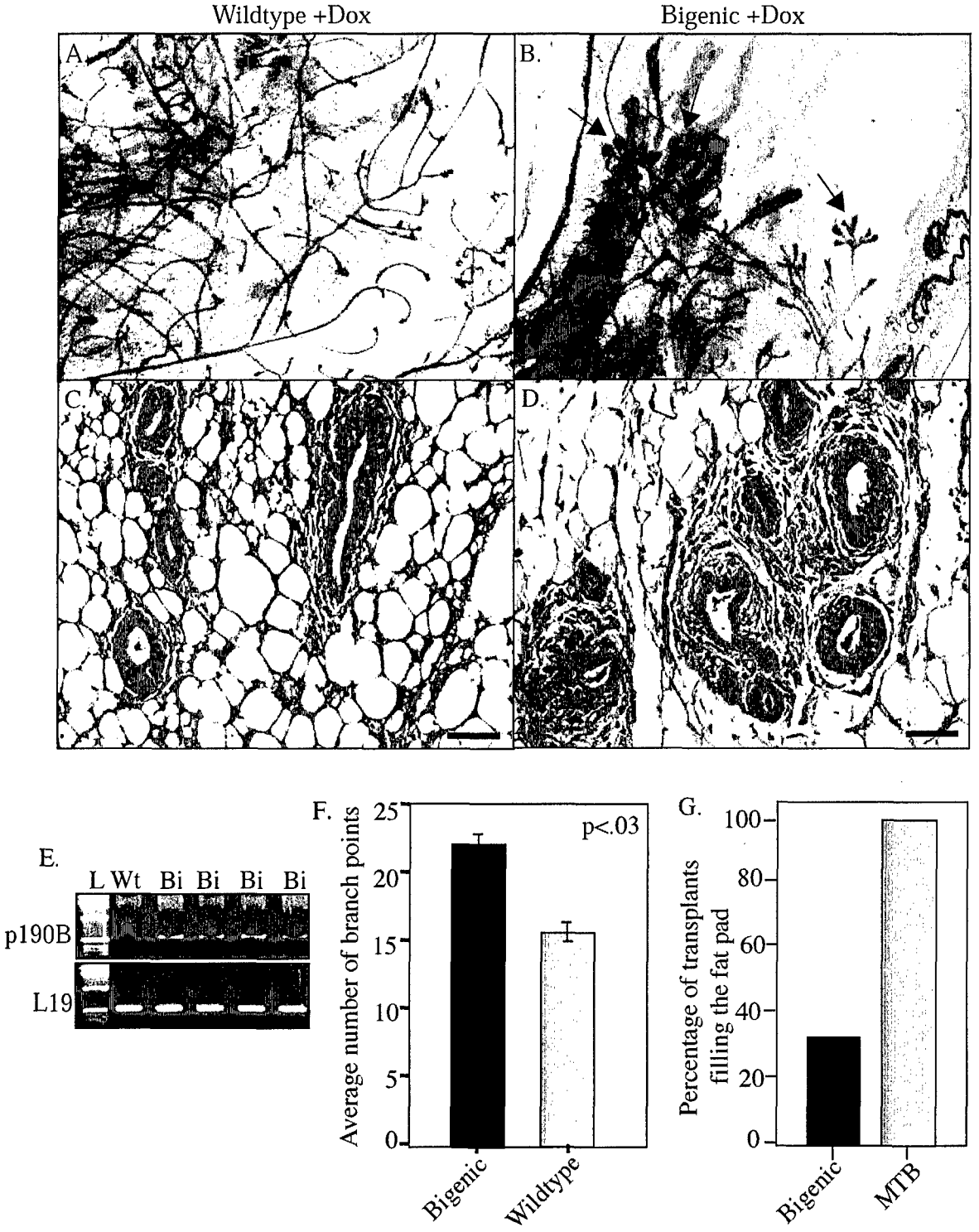

Wildtype +Dox
Bigenic +Dox
A.
B.
C.
D.
E.
L Wt Bi Bi Bi Bi
p190B
L19
F.
Average number of branch points
25
20
15
10
5
0
p<.03
Bigenic
Wildtype
G.
Percentage of transplants filling the fat pad
100
80
60
40
20
0
Bigenic
MTB

FIGURE 7